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Comparison of Reverse Hybridization, Microarray, and Sequence Analysis for Genotyping Hepatitis B Virus[▽]

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Hepatitis B virus (HBV) genotyping has become important in epidemiological and clinical diagnoses, given the relationship between the viral genotype and the progression of disease or the appearance of antiviral resistance. Since genotyping by sequence and phylogenetic analyses is not convenient in the clinical setting, we evaluated InnoLipa HBV genotyping (Innogenetics, Belgium) and an HBV DNA-Chip (bioMérieux, France) prototype assay and compared their sequencing of the gold standard S gene, using a cohort of 275 individual patient samples. All but two samples, belonging to distant and individual subgroups within a single genotype, were detected by InnoLipa HBV assay. Four samples with dual infections belonging to genotypes A and G were identified only by InnoLipa HBV assay. Using an HBV DNA-Chip assay, one sample could not be amplified due to a low viral load. Four samples were identified as genotype C and two as genotype D by sequencing but were classified as genotype A (two samples) and D (two samples) and as A (one sample) and G (one sample) by the DNA-Chip assay. In conclusion, the InnoLipa HBV genotyping strip assay detected dual infections and was an easy and quick tool for genotyping, with a sensitivity of 99.3% and a specificity of 100% compared to sequence analysis. HBV DNA-Chip assay showed a sensitivity and specificity of 97.5 and 97.8%, respectively.

It is estimated that globally, around 350 million people are chronic carriers of hepatitis B virus (HBV) (11). Two treatment strategies are available for HBV infection, based on alpha interferon treatment and the use of chain terminators (like lamivudine, adefovir, and entecavir). Molecular characterization of the virus has become increasingly important for monitoring patients (15). Recently, a correlation was found between the progression of the disease and specific genotypes of HBV (7, 9, 25). HBV can be classified into eight genotypes, named A to H (1, 22). This classification is based on the distance of the nucleotide sequence from the viral genome of 8% or greater (16, 17). These genotypes also have a distinct geographical distribution; while genotypes A and D are present mostly in Europe, Russia, India, and North Africa, genotypes B and C are more common in East Asia and Australia. Genotypes H, F, and G are present in Central and South America. Currently, there are several techniques available for genotyping HBV: sequence analysis, microarray (DNA-Chip) (23, 26), reverse hybridization (18), restriction fragment length polymorphism (5), serological assays, and genotype-specific PCR assays (6, 13). Sequence analysis is by definition the most accurate method,

but it is also the most labor intensive technique. Other techniques have the disadvantage that they are based on specific hybridization of HBV DNA, and nucleotide changes can interfere with this process and subsequent analysis. However, sequencing can always be used as a backup technology for discrepant results. An easy-to-use and rapid genotyping assay might become an important, routinely used tool for disease management in the future. In this study, we analyzed serum samples from 275 patients and compared the sequence analysis method with the HBV InnoLipa genotyping kit (Innogenetics, Gent, Belgium) and an HBV DNA-Chip assay prototype (bioMérieux, Marcy-l'Etoile, France).

MATERIALS AND METHODS

Sera were collected from an outpatient group participating in a clinical trial. Sera were stored at -20°C . HBV genotypes were analyzed in baseline samples by using sequence analysis, InnoLipa HBV genotyping strips, and HBV DNA-Chip. HBV DNA viral load was determined as described previously (10, 11).

Line probe HBV genotyping assays. For the isolation of HBV DNA from serum, a MagnaPure LC isolation station (Roche Applied Science, Almere, The Netherlands) with a modified HBV-02 protocol was used, as described previously (19, 20). An InnoLipa genotyping assay (Innogenetics, Gent, Belgium) was performed essentially as described previously (18), using AmpliTaq Gold DNA polymerase (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The hybridization, conjugate, and substrate steps were performed by using an AutoLiPa instrument (Innogenetics, Gent, Belgium) according to the manufacturer's instructions.

Sequence analysis. Isolation of the HBV DNA from serum was performed as described above. A product of 806 base pairs of pre-S and a part of the S gene were amplified with the primers ACPR (sense, 5'-CCTGCTGGTGGCTCCAG TTCCGGAACAGTA-3') and YMDD-2 (antisense, 5'-ACCCATCTTTTGT TTTGTTAGG-3'), using a PCR protocol, as follows: 10 min at 94°C , 40 cycles of 1 min at 94°C , 1 min at 52°C and 1 min at 72°C and a final elongation step of 10 min at 72°C , using AmpliTaq Gold polymerase. The amplicon was sequenced with the two above-mentioned primers and an additional YMDD-1 primer (sense, 5'-TCGCTGGATGTGTCTGCGGCGTTTAT-3'). Two microliters of

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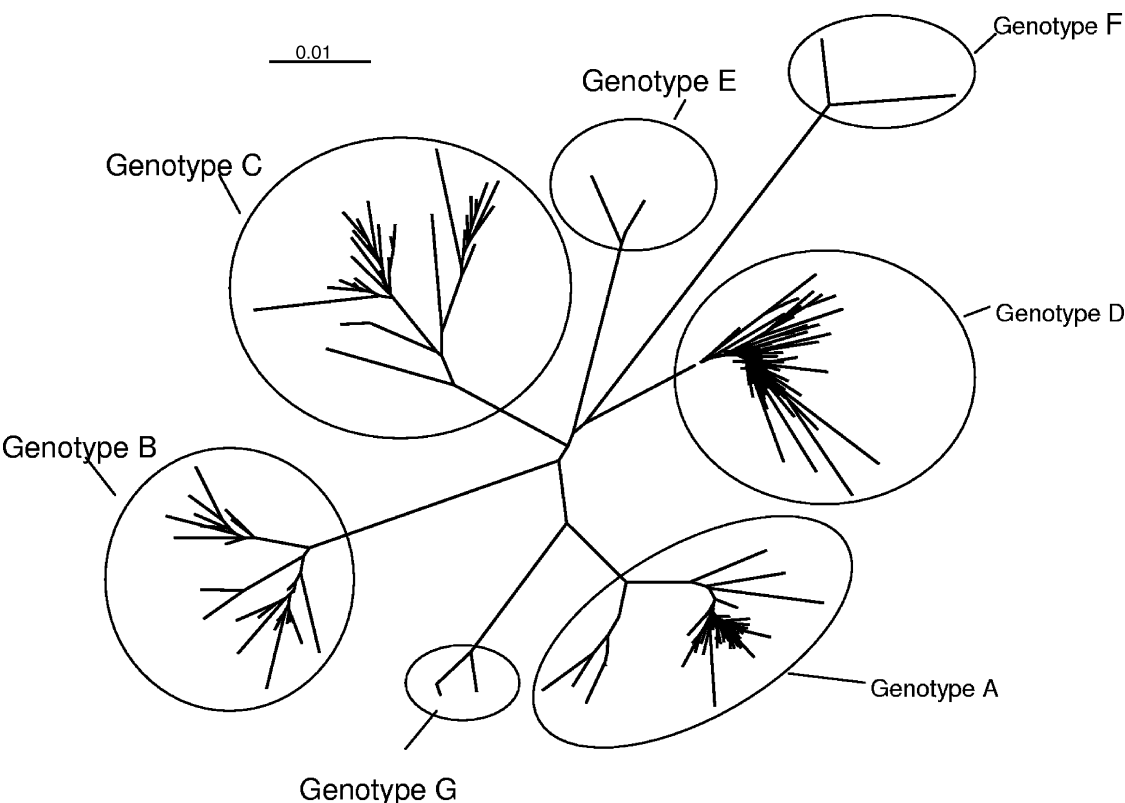


FIG. 1. Phylogenetic tree of all samples analyzed in this cohort (*n* = 275) by sequence analysis.

the amplicon was sequenced with the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The PCR products were precipitated with 80 µl NaAc buffer (3 µl 3 M NaAc [pH 4.6], 62.5 µl 96% ethanol, and 14.5 µl distilled water) and centrifuged for 1 h (2,500 × *g* at room temperature). Subsequently, the pellets were washed once with 70% ethanol and resuspended in 20 µl High Dye formamide (Applied Biosystems). The products were separated on an ABI 373 sequencer (Applied Biosystems), and the sequence data were analyzed using a Sequence Navigator software sequencer (Applied Biosystems) and SeqMan (DNASTAR, Madison, WI). Sequences of 752 base pairs and Internet reference sequences obtained from GenBank (used for comparison, Fig. 1) of different genotypes were aligned and phylogenetic relationships were calculated with bootstrapping resampling to

calculate the nodal confidence (*n* = 1,000) using Clustal W (Bioedit version 7.0.1) and Treeview version 1.6.6.

HBV DNA-Chip assay. Whole-HBV genome was amplified by a duplex PCR, which generated two products of 1.7 kb and 1.5 kb (24, 26). For amplification, 10 µl of extracted HBV DNA was amplified with primers provided by the manufacturer (bioMérieux, Marcy-l'Etoile, France), using the PCR protocol, as follows: 4 min at 94°C, 40 cycles of 20 s at 93°C, 45 s at 52.5°C, and 2 min and 30 s at 68°C, and an elongation step of 7 min at 68°C, using Fast *Taq* polymerase (Roche, Almere, The Netherlands). The PCR products were labeled for 25 min at 95°C with BioPMDAM (bioMérieux) and fragmented for 5 min at 95°C with 0.1 N HCl (3). Subsequently, the labeled PCR products were purified using a QIAquick PCR purification kit (Qiagen, Germany). Samples were hybridized on

TABLE 1. Comparison of HBV genotype analysis techniques

Sequence analysis		InnoLipa ^a				DNA-Chip ^b			
Genotype	No. of samples	Concordant results	Sensitivity (%)	False-positive results	Specificity (%)	Concordant results	Sensitivity (%)	False-positive results	Specificity (%)
A	92	92 ^c	100	0	100	92	100	3	96.8
B	26	25	96.2	0	100	25	96	0	100
C	40	39	97.5	0	100	36	90	0	100
D	109	109	100	0	100	107	98.2	2	98.2
E	3	3	100	0	100	3	100	0	100
F	2	2	100	0	100	2	100	0	100
G	3	3 ^c	100	0	100	3	100	1	75
Total	275	273	99.3	0	100	268	97.5	6	97.8

^a InnoLipa produced two negative results and four mixed-population results. Although bands were visible on the gel, no bands were visible on the blot. The samples were classified as genotype B and C by sequence analysis.

^b DNA-Chip analysis produced one negative result. This sample was classified as genotype B by sequence analysis; as the viral load was 9.28 × 10² copies/ml, we were unable to amplify the viral DNA with the HBV DNA microarray PCR.

^c One sample was classified as genotype G, and three samples were classified as genotype A by sequence analysis.

the HBV DNA-Chip prototype and stained with streptavidin-phycoerythrin conjugate on a GeneChip fluidics station 400 (Affymetrix, Santa Clara, CA). The HBV DNA-Chips were scanned on an HP Gene Array scanner (Affymetrix) and were analyzed by using DNA-Chip evaluation software (bioMérieux).

RESULTS

A total of 275 serum samples were analyzed by phylogenetic analysis (Fig. 1), InnoLipa HBV genotyping assay, and HBV DNA-Chip analysis (Table 1).

Patients included in this study came from a wide geographic distribution, including 38% from northwestern Europe, 12% from Eastern Europe, 32% from the Mediterranean, 11% from East Asia, and 6% from North America. Caucasians were infected mainly with genotype A or D (43% and 52%, respectively) and Asians with genotype B or C (40% and 58%, respectively). In different areas of Europe, HBV genotypes were distributed as follows. In northwestern Europe, we found genotype prevalences as follows: A, 51%; B, 7%; C, 10%; and D, 24%. For the Mediterranean area, genotype prevalences were as follows: A, 6%; and D, 93%; and for Eastern Europe, the genotype prevalences were A, 94%; and D, 6%. The mean log HBV-DNA viral loads were 9.05 copies/ml for genotype A, 8.52 for genotype B, 8.57 for genotype C, and 9.33 for genotype D. HBV DNA levels were significantly higher in sera of patients infected with genotype D than with genotypes B and C ($P < 0.003$).

All but one sample (9.28×10^2 copies/ml) had a copy number above 1,000 copies/ml, and although the limit of detection of both sequence analysis and InnoLipa was around 1,000 copies/ml, all samples analyzed by both methods gave a band on agarose gel, which in our hands, has a detection limit of around 400 copies per ml (data not shown). The InnoLipa HBV genotyping assay was able to identify the same genotype as the sequencing method in 99.3% of the analyzed samples. However, in two samples, no bands were visible on an InnoLipa blot (HBV DNA viral load of 4.20×10^9 and 1.23×10^9 copies/ml, respectively).

As the detection limit of an HBV DNA-Chip assay is around 5,000 copies/ml, we were not able to detect HBV DNA from one of the samples (HBV DNA viral load of 9.28×10^2 copies/ml) with the HBV microarray PCR method. The HBV DNA-Chip assay was able to identify the genotypes in 97.5% of all analyzed samples; one sample was negative with the PCR, while six samples initially had a result that differed from that obtained by sequencing. Of these six samples, four were identified as genotype C but were classified by DNA-Chip analysis as genotypes A (two samples) or D (two samples); the other two samples were classified by DNA-Chip analysis as genotypes A and G but were classified as genotype D by sequence analysis. Retesting was undertaken with four of these six samples and gave the same result as sequencing, leaving two discrepant results (one genotype D and one genotype C, classified by DNA-Chip analysis as genotypes G and D, respectively).

A perfect concordance of InnoLipa HBV genotyping assay was found in a comparison with sequence analysis for genotypes A, D, E, and F or G (Table 1). The InnoLipa HBV genotyping assay can detect minor virus subpopulations of different genotypes (Fig. 2), whereas minor populations are more difficult to interpret by sequence analysis alone. For

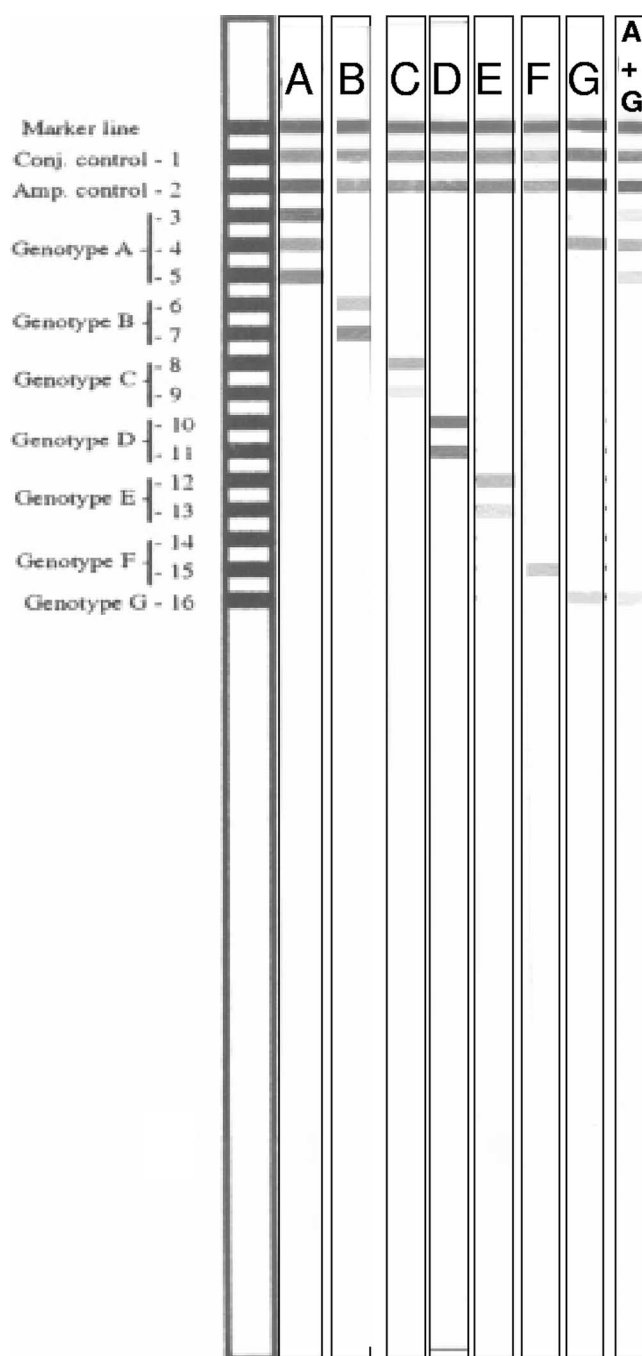


FIG. 2. InnoLipa HBV genotyping strips. On the left is a reference card supplied by the manufacturer, then seven strips with genotypes A through G and an extra strip for a double infection of genotype A and G.

genotype G, two samples were classified by both assays as genotype G alone. One sample was classified by sequence analysis as genotype G, whereas the InnoLipa HBV genotyping assay could detect a dual infection of genotypes A and G. Two samples that were classified as genotype D by sequence analysis had to be retested with an InnoLipa HBV genotyping assay. The second time, two very light bands were visible on the InnoLipa blot, classifying these samples as genotype D. The

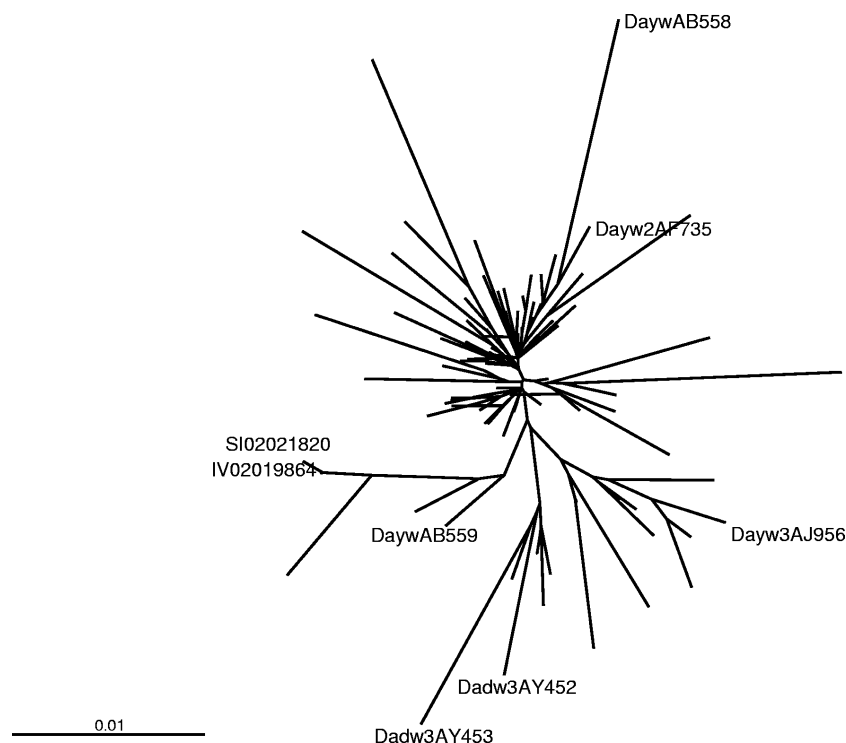


FIG. 3. Phylogenetic tree of all samples classified as genotype D by sequence analysis, including the Internet reference sequences. The two numbers shown at the left belong to two samples that the InnoLipa HBV genotyping strip had difficulties identifying. Only the second hybridization had two very light bands.

sequence alignment of these two samples (derived from two individual patients) with the other genotype D samples showed that these samples belonged to a subgroup (Fig. 3). Two samples could not be classified using an InnoLipa HBV genotyping assay: one sample which was classified by sequence analysis as

genotype B (Fig. 4) and one as genotype C (Fig. 5). This resulted in a concordance of an InnoLipa HBV genotyping assay and a sequence analysis of 96.2% and 97.5% for genotype B and C, respectively. HBV DNA-Chip assay achieved a 100% sensitivity with

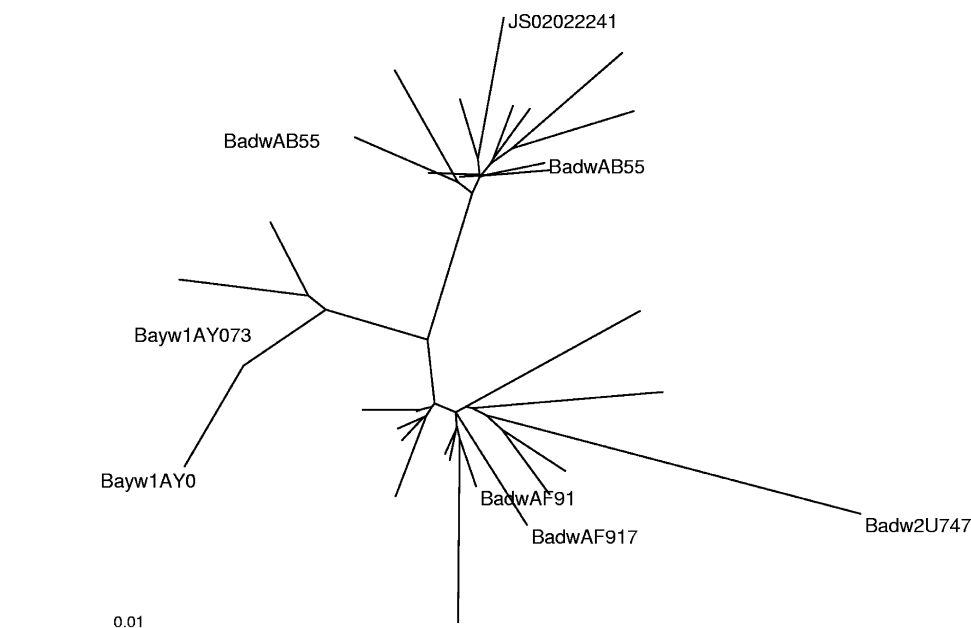


FIG. 4. Phylogenetic tree of all samples classified as genotype B by sequence analysis, including the Internet reference sequences. The designation number shown at the top belongs to the sample that could not be classified with the InnoLipa HBV genotyping strip.

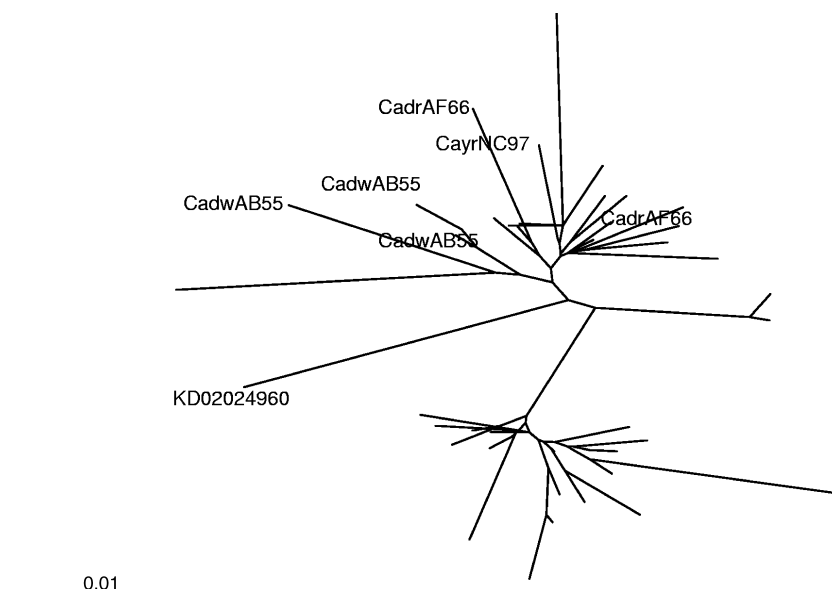


FIG. 5. Phylogenetic tree of all samples classified as genotype C by sequence analysis, including the Internet reference sequences. The designation number shown at the left belongs to the sample that could not be classified with the InnoLipa HBV genotyping strip.

samples that by sequence analysis, were identified as genotypes A, E, and F or G (Table 1). However, for genotype A, we found a specificity of 96.8%, due to three samples which were classified as genotype C (two samples) and D (one sample) by sequence analysis and InnoLipa. These three samples were analyzed twice with the HBV DNA-Chip assay, and upon repeating, the genotypes found by sequencing and InnoLipa were also identified by DNA-Chip analysis. Similarly, the specificities with samples identified as genotypes D and G were, respectively, 98.2% (two false-positive results were identified as C by sequencing and InnoLipa) and 75% (one false-positive result was identified as genotype D by sequencing and InnoLipa). Only one sample could be retested, and the genotype found by sequencing and InnoLipa was identified correctly upon repetition.

An HBV DNA-Chip PCR could not amplify HBV DNA from one sample, classified as genotype B by sequence analysis, as the viral load was below the detection limit (9.28×10^2 copies/ml). This resulted in a sensitivity level for the HBV DNA-Chip assay of 96% for genotype B. HBV DNA-Chip assay had a lower discrimination for genotype C; the sensitivity with samples identified as genotype C by sequence analysis was only 90%. In total, four samples determined as genotype C by sequence analysis could not be classified correctly with an HBV DNA-Chip assay. Two samples were classified as genotype A and two samples as genotype D. After repeating the analysis, three samples were classified correctly, whereas a fourth sample could not be retested. Although the sensitivity was better than that for genotype C (90.0%), the sensitivity for genotype D was 98.2%.

DISCUSSION

In this paper, we describe the comparison of three HBV genotyping assays. Sequence analysis was used as the gold standard and was compared to InnoLipa HBV genotyping

assay and a prototype HBV DNA-Chip assay. Using sequencing as the gold standard implies that it can be used to determine the most abundant genotype present in the sample. However, our data demonstrate that sequence analysis is not the method of choice to determine the presence of double infections, as the quality of the sequence data themselves and the experience of the interpreter cannot be standardized and influence the conclusion.

Only two out of 275 samples could not be classified using HBV InnoLipa; these samples belonged to genotypes B and C, according to sequence analysis. Most likely, this was due to mismatches in the probes used to anneal to these HBV sequences. The assay was highly sensitive (96% and 98% for genotypes B and C, respectively, and 100% for the other genotypes) and specific (100%) for all genotypes. In contrast to sequence analysis, an advantage of the InnoLipa assay is the ability to detect dual infections. A prototype of the HBV DNA-Chip assay was tested during this evaluation. Apart from the future updates of the sequence patterns that will become available, the DNA chip used in this study represents the current status for HBV genotyping with this technology. For this assay, the analyses resulted in a correct classification of the HBV genotype in 97.5% of the samples. Four out of six incorrectly typed samples were classified by sequence analysis as genotype C but as genotypes A (two samples) and D (two samples) using DNA-Chip analysis. Misclassification was the main problem of the prototype HBV chip and may be due to an under-representation of genotype C sequences in the alignment used for probe sequence design of the HBV DNA-Chip. The other two incorrectly typed samples were classified by the DNA-Chip as genotypes A and G, but by sequence analysis, they were classified as genotype D. Furthermore, the HBV DNA-Chip assay is rather labor intensive; only four samples can be hybridized and detected simultaneously.

It is clear that genotyping HBV has a place within the diag-

nostic repertoire of the clinical laboratory. There are more and more reports describing differences in clinical outcome of the infection for different genotypes (14), as well as differences in outcome related to antiviral treatment with nucleoside analogues. Patients infected with genotype C or D, for instance, have a lower response to interferon treatment compared to those infected with genotypes A or B (9, 10). There is a relationship found between the HBV genotype and the prevalence of hepatocellular carcinoma, as well as a prognosis for disease outcome, which is better for genotype B compared to that of genotype C (4, 12, 21). Understanding the relationship between the genotype and outcome of disease, as well as treatment, will improve patient management in the future.

Several technologies are available for easy and rapid identification of HBV genotypes. Although sequences provide the most correct information, the easy-to-use InnoLipa assay has some advantages. The technology has been proven for the typing of HCV and is easy to perform, but most interestingly, it gives indications of double infections in a given sample. There are reports indicating that double infections occur (2, 8), and although this occurs in a minority of patients, this information may be valuable in guiding treatment regimens.

DNA-Chip technology is currently not used routinely in a clinical laboratory, although the potential is enormous. Aside from the information relating to the HBV genotype, more sequence patterns related to antiviral resistance or promoter sequence variation can be located on a single chip. Even sequences for more viruses or for the human genome could be made available. However, it should also be noted that an easy-to-use assay for this technology is not yet available and that the number of samples that may be analyzed was limited to 8 to 12 per day.

In this study, we performed an extensive evaluation of technologies currently available for HBV genotyping. For routine use, the InnoLipa assay has several advantages, while sequencing technologies should be used to determine the few samples not detected by this assay. Implementing this technology into routine clinical diagnostics will definitely be of value to the determination of the relationship between HBV genotype and the progression of disease, disease outcome, and the response to treatment options (like interferon or nucleoside analogues).

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